Viscoelasticity of oral biofilms and antimicrobial penetration - an in vitro and in vivo study -

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CHAPTER 4

Viscoelasticity of Oral Biofilms and Antimicrobial Penetration of Different Commercial Mouthrinses *In Vitro*

ABSTRACT

In previous chapters, we described a relation between the viscoelastic relaxation of oral biofilms and the penetration of chlorhexidine, for *in vitro* and *in vivo* grown biofilms. In this chapter, we confirmed that also other, commercially available mouthrinses followed this relation. Importantly, in this chapter we applied oral biofilms grown statically in a well system, but not in a constant depth film fermenter or parallel plate flow chamber. Biofilms grown statically in a well, have relatively high fast relaxation components, attesting to a large water content of the biofilms.

INTRODUCTION

In previous chapters, we demonstrated that stress relaxation of oral biofilms relate with the penetration of chlorhexidine (see Fig. 1), both for *in vitro* biofilms grown in a constant depth film fermenter or in a parallel plate flow chamber, as well as for *in vivo* formed biofilms. Stress relaxation analysis of oral biofilms identified a fast, intermediate and slow response to an induced deformation, corresponding with the flow of water and extracellular polymeric substances, and bacterial re-arrangement, respectively. Penetration of chlorhexidine into biofilms increased with the

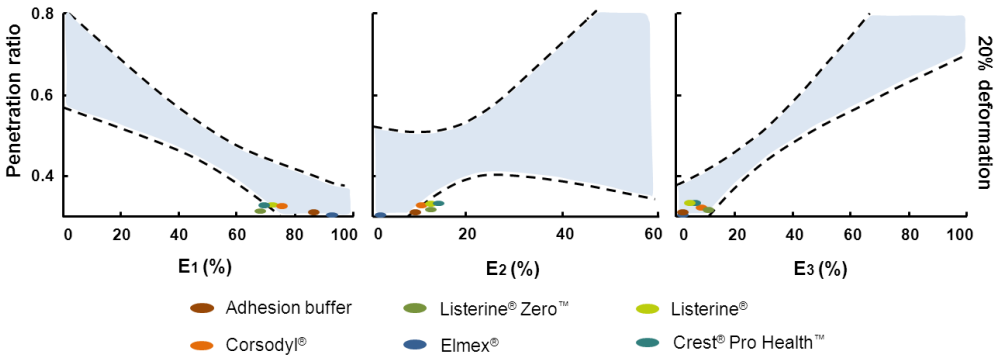

Figure 1 Penetration of different antimicrobials from commercially available mouthrinses as a function of the relative importance of the different Maxwell elements describing the viscoelasticity of *in vitro* oral biofilms, grown in wells (for details see chapter 2). Shaded areas indicate the 95% confidence intervals of these relations obtained for the penetration of chlorhexidine into *in vitro* biofilms grown in a parallel plate flow chamber and constant depth film fermenter.

increasing relative importance of the slow and decreasing importance of the fast relaxation element. Involvement of a slow relaxation element suggests that biofilm structures allowing extensive bacterial re-arrangement after deformation are more open, allowing better antimicrobial penetration. Involvement of a fast relaxation element

suggests that water dilutes the antimicrobial upon penetration to an ineffective concentration in deeper layers of the biofilm. It is unknown however, whether this relation holds for other oral antimicrobials as well.

Therefore, the aim of this chapter is to investigate whether also penetration of other commercially available mouthrinses follow the same relation with viscoelasticity as demonstrated previously for chlorhexidine.

MATERIALS & METHODS

Bacterial strains and growth conditions

Streptococcus oralis J22 grown on blood agar plates, were used to inoculate 10 mL Brain Heart Infusion broth (BHI, Oxoid Ltd., Basingstoke, Hampshire, UK) with yeast extract (37.0 g/L BHI, 5.0 g/L yeast extract, pH 7.3) and were cultured for 24 h at 37°C in ambient air. The culture was used to inoculate 200 mL BHI and grown for 16 h. Bacteria were harvested by centrifugation at 870 g, 10°C for 5 min and washed twice in sterile adhesion buffer (50 mM potassium chloride, 2 mM potassium phosphate, 1 mM calcium chloride, pH 6.8). The bacterial pellet was suspended in 10 mL sterile adhesion buffer and sonicated intermittently in an ice-water bath for 3 × 10 s at 30 W (Vibra cell model 375, Sonics and Materials Inc., Newtown, CT, USA) to break bacterial chains and clusters, after which bacteria were resuspended in adhesion buffer. A concentration of 3×10^8 bacteria/mL was used for 24 h static biofilm growth in ambient air at 37°C.

Biofilm formation

Biofilms were grown on glass slides (water contact angle 7 ± 3 degrees) in a petri dish after the adsorption of a salivary conditioning film from

reconstituted human whole saliva for 14 h at 4°C under static conditions. Reconstituted human whole saliva was obtained from a stock of saliva from at least 20 healthy volunteers of both genders, collected into ice-cooled beakers after stimulation by chewing Parafilm®, pooled, centrifuged, dialyzed, and lyophilized for storage. Prior to lyophilization, phenylmethylsulfonylfluoride was added to a final concentration of 1 mM as a protease inhibitor in order to reduce protein breakdown. Freeze-dried saliva was dissolved in adhesion buffer (1.5 g/L). All volunteers gave their informed consent to saliva donation, in agreement with the guidelines set out by the Medical Ethical Committee at the University Medical Center Groningen, Groningen, The Netherlands (letter 06-02-2009).

For biofilm formation, a saliva coated microscope glass slide was immersed in 25 mL bacterial suspension in a sterilized petri dish for 30 min at room temperature. Subsequently, the slide was immersed in 25 mL adhesion buffer in order to remove non-adhering bacteria. Next, the slide was incubated with 25 mL BHI at 37°C for 24 h statically in ambient air.

Low load compression testing and Maxwell analysis

The thickness and stress relaxation of the biofilms were measured with a low load compression tester (LLCT). The LLCT was described previously (Paramonova *et al.*, 2009). Stress relaxation was monitored after inducing a 20% deformation of the biofilms within 1 s, and held constant for 100 s (see Fig. 1A, chapter 2). Each deformation was induced 3 times at different locations on the same biofilm. Stress relaxation as a function of time was subjected to a Maxwell analysis as described in chapter 2, yielding a fast, intermediate and slow relaxation element.

Penetration of mouthrinses into biofilms

Five different mouthrinses were commercially obtained for this study (see Table 1). Adhesion buffer was used as a negative control. Biofilms were exposed to 25 mL of a mouthrinse for 30 s and subsequently immersed in 25 mL adhesion buffer for 5 min after which biofilms were stained with live/dead stain (*Bac*Light™, Invitrogen, Breda, The Netherlands) to visualize live and dead bacteria in order to allow calculation of the penetration ratio (see chapter 2). Note that whereas in previous chapters a dead/live ratio R/G equal to 1.5 was taken, the aqueous nature of statically grown biofilms necessitated the use of R/G equal to 1.0 in order to distinguish between penetration by different mouthrinses (see also Fig. 2).

Table 1 Overview of the commercially available mouthrinses used in this study, together with the main active components and manufacturers.

Mouthrinse	Main active components	Manufacturer
Corsodyl®	chlorhexidine digluconate (CHX, 20,000 ppm) and ethanol (11.8%)	SmithKline Beecham Consumer Brands B.V., Rijswijk, The Netherlands
Listerine® Cool Mint	essential oils, thymol and ethanol (21.6%)	Pfizer Consumer Healthcare, Morris Plains, NJ, USA
Listerine® Zero™	essential oils, thymol and sodium fluoride (220 ppm)	Pfizer Consumer Healthcare, Morris Plains, NJ, USA
Crest® Pro Health™	cetylpyridinium chloride (CPC, 700 ppm)	Procter & Gamble, Cincinnati, USA
Elmex®	aminefluoride and sodium fluoride (total fluoride 250 ppm)	Gaba, Lorrach, Germany

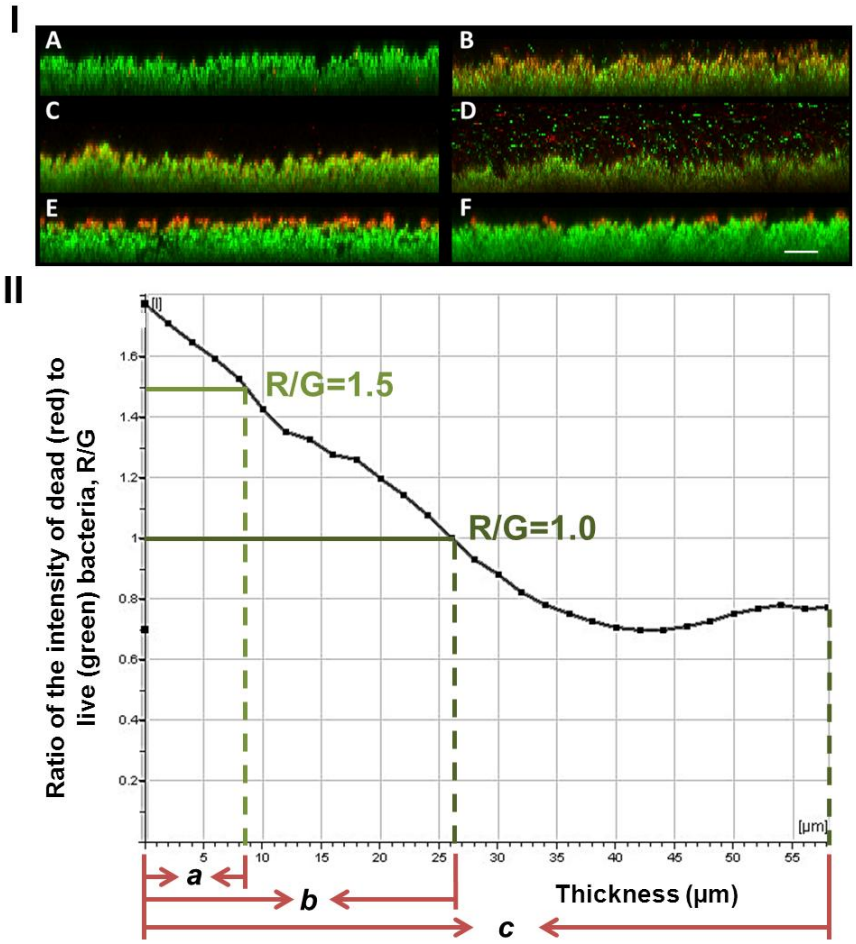

Figure 2 Mouthrinse penetration into *in vitro* oral biofilms of *S. oralis* J22 formed statically in wells and the calculation of the penetration ratio. **I.** Representative confocal laser scanning microscope images (cross sectional view) of *S. oralis* biofilms after 30 s exposure to adhesion buffer or commercially available mouthrinses with different antimicrobials. **(A)** Adhesion buffer (negative control); **(B)** Corsodyl®, containing chlorhexidine and ethanol; **(C)** Listerine®, containing essential oils, thymol and ethanol; **(D)** Listerine® Zero™, containing essential oils, thymol and sodium fluoride; **(E)** Crest® Pro Health™, containing cetylpyridinium chloride; **(F)** Elmex®, containing aminefluoride and sodium fluoride. Scale bar represents 40 µm. **II.** Red to green intensity ratio (R/G), denoting the ratio of dead to live microorganisms in a biofilm *versus* the thickness of the biofilm. *a* denotes the dead band thickness when R/G is taken 1.5, while *b* is the dead band thickness resulting from R/G equal to 1.0. *c* is the total biofilm thickness.

Statistical analysis

Statistical analysis was performed with SigmaPlot software (version 11.0, systat software, Inc., California, USA). Differences in biofilm thickness and viscoelasticity were evaluated after testing for normal distribution and equal variance of the data. If data failed one of these tests, a Mann-Whitney Rank Sum test was used to determine statistical significance; otherwise a Student *t*-test was applied.

RESULTS & DISCUSSION

Biofilms of *S. oralis* J22 grown statically in wells reached a thickness of 41 to 47 µm (see Table 2), while exposure to mouthrinses had no effect on their thickness ($p > 0.05$). Compared with the biofilms studied in previous chapters, biofilms grown statically in wells had a high fast relaxation component (E_1), attesting to a high amount of water in the biofilms. Accordingly, the slow relaxation component (E_3) was low (see Fig. 1). As a consequence, penetration as evidenced by analysis of the live and dead ratio at R/G = 1.5 was extremely low for all mouthrinses due to rapid dilution to ineffective concentration in water-rich biofilms. Nevertheless, this echoes our previous discovery on the relaxation-structure-composition relation: the fast component of a biofilm negatively relates with antimicrobial penetration. Herewith, this chapter provides an important extension to the previous ones, demonstrating:

1. That the impact of viscoelastic relaxation parameters on antimicrobial penetration is not only valid for chlorhexidine, but also for other oral antimicrobials.

2. That the relation between viscoelastic relaxation parameters and antimicrobial penetration also hold for highly aqueous biofilms, grown statically in wells.

Considering that the different mouthrinses evaluated in this study contain chemically highly diverse components, it was anticipated that their penetration would be different. In Fig. 1, we employ an R/G ratio equal to 1.5, in order to make the analysis comparable to the one in previous chapters. However, in order to reveal differences in penetration between the different mouthrinses, i.e. their active components, we here also employ a ratio R/G equal to 1.0, yielding significant differences in penetration of the different antimicrobials (see Table 2).

Table 2 Biofilm thickness and penetration ratio of mouthrinses. Note that biofilm thickness was not affected by exposure to any of the mouthrinses.

Mouthrinse	**Thickness (μm)**	**For R/G = 1.5**	**For R/G = 1.0**
Adhesion buffer	42 ± 9	0.01 ± 0.04	0.03 ± 0.06
Corsodyl®	46 ± 8	0.06 ± 0.08	0.26 ± 0.14*
Listerine®	45 ± 7	0.07 ± 0.09	0.18 ± 0.18
Listerine® Zero™	41 ± 5	0.05 ± 0.08	0.15 ± 0.12
Crest® Pro Health™	44 ± 6	0.07 ± 0.12	0.23 ± 0.14*
Elmex®	47 ± 3	0.00 ± 0.00	0.06 ± 0.06

* $p < 0.05$

Chlorhexidine and cetylpyridinium chloride both showed similar and relatively high penetration coefficient, followed by the essential oils (regardless of the absence or presence of alcohol). Aminefluoride remained to have low penetration, despite the use of a lower R/G ratio.

Interestingly, cetylpyridinium and aminefluoride both are positively charged in aqueous solutions, yet their penetration is highly different. This shows that not only the chemical nature of the antimicrobial determines penetration, but also their concentration (Da Silva *et al.*, 2013; Corvec *et al.*, 2013). Aminefluoride is present in its mouthrinse formulation in a concentration less than 250 ppm (Marinho *et al.*, 2004; Xu *et al.*, 2012), while 700 ppm cetylpyridinium is present in Crest® Pro Health™ causing its higher penetration. We speculate the difference was caused by their main active components. Chorhexidine also has a positive charge, although in a conjugate bond. However, despite its higher concentration in Corsodyl® with respect to the concentration of cetylpyridinium in Crest® Pro Health™, it penetrates to the same extent, which is probably due to the presence of alcohol in Corsodyl®. Both variants of Listerine® (Listerine® Cool Mint *versus* Listerine® Zero™) have similarly low penetration ratios, despite the fact that there is no ethanol in Listerine® Zero™. We speculate that the hydrophobicity of the essential oils is a dominant factor controlling penetration in aqueous biofilms.

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